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PRINCIPAL INVESTIGATOR: Linara S. Axanova, M.S.

CONTRACTING ORGANIZATION: Wake Forest University Health Sciences
Winston-Salem, NC 27157

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14. ABSTRACT 1-alpha, 25-dihydroxy vitamin D3 (1,25(OH)2D3) elicits antiproliferative effects in a variety of cancer cell types including prostate cells while the PI3K/AKT pathway stimulates pro-survival signals. The antiproliferative effect of 1,25(OH)2D3 almost universally involves upregulation of p21 and/or p27, while activation of PI3K/AKT downregulates p21 and p27 levels. We hypothesized that inhibition of the PI3K/AKT pathway synergizes with the antiproliferative signaling of 1,25(OH)2D3. We demonstrate that pharmacological inhibitors of PI3K or Akt restored sensitivity to antiproliferative effects of 1,25(OH)2D3 in 1,25(OH)2D3-insensitive cells and synergized to inhibit the growth of Pten-/- mouse prostatic epithelial cells, DU145, LNCaP and primary human prostate cancer cell strains. The inhibitors used included API-2 (Triciribine) and GSK690693 which are currently in clinical trials for treatment of cancer. The combination of 1,25(OH)2D3 and AKT inhibitor API-2 led to a cooperative increase in G1 arrest and in the induction of senescence. AKT is commonly activated by PTEN loss, therefore we evaluated the role of Pten in responsiveness to 1,25(OH)2D3 using shRNA knockdown and by in vitro knockout of Pten. We found that Pten status did not affect responsiveness to the antiproliferative action of 1,25(OH)2D3. These findings provide the rationale for the development of therapeutic interventions utilizing 1,25(OH)2D3 or its analogs combined with inhibition of PI3K/AKT for the treatment of prostate cancer.					
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Introduction

Prostate cancer (CaP) is the most commonly diagnosed cancer and the second most common cause of cancer death in American men (1). It has been suggested that the development of clinical prostate cancer may be associated with vitamin D₃ deficiency (2, 3).

Vitamin D is a hormone that can be obtained from the diet or produced endogenously by a series of reactions that culminates in the most active metabolite of vitamin D, 1 α , 25(OH)₂-vitamin D₃ (1,25(OH)₂D₃), also called calcitriol. 1,25(OH)₂D₃ elicits antiproliferative effects in a variety of cancer cell types including cell lines derived from prostate (4, 5). The ability of 1,25(OH)₂D₃ to inhibit prostate growth was also demonstrated in primary prostatic cells from histologically normal, benign prostatic hyperplasia, and prostate cancer specimens (6), in multiple prostate cancer cell lines (7-9), in xenograft models of prostate cancer (10, 11) as well as in Dunning rat prostate model (12). The anticancer mechanisms of 1,25(OH)₂D₃'s action include induction of cell cycle arrest, promotion of differentiation, inhibition of proliferation and angiogenesis, as well as inhibition of invasive and migratory potential of cancer cells [reviewed in (13)].

Classical actions of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR) which is a member of a superfamily of nuclear steroid hormone receptors. Upon 1,25(OH)₂D₃ binding VDR translocates to the nucleus, dimerizes with retinoid X receptor (RXR) and modulates the expression of target genes. Although a number of 1,25(OH)₂D₃ responsive genes are known, the exact mechanism of growth regulation by 1,25(OH)₂D₃ is not completely defined; however, an increase in p21 and/or p27 is an almost universal feature (4).

One of the central contributing factors which facilitates the survival of prostate cancer cells is attributed to the phosphoinositol-3 kinase PI3K-AKT pathway. Activated AKT phosphorylates a host of proteins that affect cell growth, cell cycle entry, and cell survival. The AKT pathway presents an attractive target for anticancer therapies and several AKT inhibitors have been developed that demonstrate anticancer activity in preclinical and clinical studies (14). In prostate cancer, activation of AKT occurs most frequently due to the loss of tumor suppressor phosphatase and a tensin homologue deleted in chromosome ten (PTEN)(15-17). Loss of PTEN protein occurs in 20% of primary prostate tumors and this loss is highly correlated with advanced tumor grade and stage with 50% of metastatic tumors exhibiting a loss of PTEN protein (18). Moreover, loss of heterozygosity (LOH) is found in 20% to 60% of metastatic tumors (19). Data suggests that advancing disease is associated with a progressive loss of PTEN or an accumulation of mutations in the PTEN gene. Loss of PTEN and activation of AKT has been shown to downregulate the expression of p21 and p27 by a number of mechanisms (20-25).

In our preliminary data we had shown that *Pten* WT mouse prostatic epithelial cells (MPEC) exhibit significant levels of growth-inhibition in response to the presence of 1,25(OH)₂D₃, whereas growth of *Pten*^{-/-} MPECs in which *Pten* was lost *in vivo* are not affected by 1,25(OH)₂D₃. Pretreatment of *Pten*^{-/-} MPECs with PI3K/AKT inhibitors resulted in the partial restoration of sensitivity to 1,25(OH)₂D₃. Given our preliminary findings and the existing data contained within recent literature, we proposed the hypothesis that **1,25(OH)₂D₃-mediated growth inhibition of prostate epithelial cells is antagonized by PTEN loss.**

Body

SOW TASK 1:

In our preliminary data we had shown that *Pten* WT mouse prostatic epithelial cells (MPEC) exhibit significant levels of growth-inhibition in response to the presence of $1,25(\text{OH})_2\text{D}_3$, whereas growth of *Pten*^{-/-} MPECs in which *Pten* was lost *in vivo* are not affected by $1,25(\text{OH})_2\text{D}_3$. In SOW Task 1 we proposed to evaluate the role of *Pten* in $1,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition in more controlled settings. Thus, we generated MPEC in which *Pten* expression was suppressed using an shRNA approach. MPEC WFU3 were infected with either lentivirus expressing scrambled shRNA or with lentivirus expressing shRNA targeting *Pten* (the details of the method is described in the Materials and Methods section of the attached manuscript). Single cell clones of each of the cell types were established and *Pten* status was verified by Immunoblot (Fig. S5A of the attached manuscript). WFU3 control MPEC clones and WFU3 MPEC clones with *Pten* knocked down to undetectable levels were treated with increasing doses of $1,25(\text{OH})_2\text{D}_3$ as was proposed in SOW Task 1C. There was no significant difference in the response to $1,25(\text{OH})_2\text{D}_3$ based on *Pten* status; both cell types remained $1,25(\text{OH})_2\text{D}_3$ -sensitive (Fig. 4B). This suggested that suppression of *Pten* expression by shRNA was not sufficient to abrogate the growth-inhibitory qualities of $1,25(\text{OH})_2\text{D}_3$ in MPEC.

Since gene suppression using an shRNA approach has the potential disadvantages of incomplete suppression of target gene expression and also possible off-target effects, we sought to establish a cell line with acute *in vitro* deletion of *Pten* as was proposed in SOW Task 1A. Thus, we isolated *Pten*^{lox/lox} MPEC from prostates of *Pten*^{lox/lox} Cre-recombinase negative animals and infected them with lentivirus expressing self-deleting Cre-recombinase (for more details see Material and Methods section of the attached manuscript). PCR and Immunoblot analysis of the deletion demonstrated the absence of *Pten* expression in the cells infected with Cre-recombinase (Fig. S5B and S5C).

As proposed in SOW Task 1C the control *Pten*^{lox/lox} MPEC and *Pten*^{-/-} (*Pten*^{lox/lox} infected with Cre-recombinase lentivirus) cells were treated with increasing concentrations of $1,25(\text{OH})_2\text{D}_3$ and cell viability was evaluated by trypan blue exclusion assay as well as by clonogenic survival. We observed that *in vitro* acute loss of *Pten* did not significantly affect the ability of $1,25(\text{OH})_2\text{D}_3$ to inhibit the growth of the cells (Fig. 4C). These results were confirmed by clonogenic assays (Fig. S6, S7). Together, these data demonstrate that while *in vivo* loss of *Pten* was associated with resistance to $1,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition of MPEC, *in vitro* suppression or loss of *Pten* in MPECs was not associated with increased resistance to $1,25(\text{OH})_2\text{D}_3$.

As our hypothesis that *Pten* status determines the ability of prostate cells to be growth inhibited by $1,25(\text{OH})_2\text{D}_3$ was not confirmed we did not continue with SOW Task 1D which was intended to determine the role of lipid and protein phosphatase activity of *Pten* in $1,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition. For the same reason we did not proceed with the SOW Task 1E which aimed to establish the role of PTEN loss in response of human prostate cancer cells to $1,25(\text{OH})_2\text{D}_3$.

As it was mentioned above our preliminary pretreatment of *Pten*^{-/-} MPECs with PI3K/AKT inhibitors resulted in the partial restoration of sensitivity to 1,25(OH)₂D₃. In addition, activation of AKT has been shown to downregulate the expression of p21 and p27 by a number of mechanisms (20-25) while the antiproliferative effects of 1,25(OH)₂D₃ involve upregulation of p21 and/or p27 (4), we next hypothesized that pharmacological inhibitors of AKT will cooperate with the antiproliferative actions of 1,25(OH)₂D₃ in prostate cancer cells. These experiments were not proposed in Statement of Work submitted to DOD; however, have a straight relevance to the research project as well as high importance due to the fact that a number of AKT inhibitors as well as 1,25(OH)₂D₃ or its analogs are currently in clinical trials for treatment of cancer. The potential existence of synergism between these compounds could significantly enhance the therapeutic efficacy of these clinically relevant drugs while reducing the potential side effects.

We first utilized a pharmacological inhibitor of PI3K/AKT, LY294002 (27). Cells were treated with increasing doses of 1,25(OH)₂D₃ or LY294002 or with multiple combinations of the two compounds and the number of viable cells was assessed. Synergism was assessed with the Chou-Talalay method (28) in which the combination index (CI) values < 1 indicates presence of synergism.

We found that LNCaP cells, which lack functional PTEN, and are moderately sensitive to growth inhibition by 1,25(OH)₂D₃, demonstrated statistically significant synergism between 1,25(OH)₂D₃ and LY294002 (Fig. 1A, Table 1, and Fig S1A).

DU145 cells (wild-type PTEN) are known to be resistant to the antiproliferative effects of 1,25(OH)₂D₃ (29). While 100 nM 1,25(OH)₂D₃ did not inhibit growth of DU145 cells, pretreatment of DU145 cells with 5 μM LY294002 sensitized the cells to 1,25(OH)₂D₃ treatment leading to a reduction in the number of viable cells to 40% compared to treatment with LY294002 alone (Fig 1B and S1B). We found significant synergism across multiple doses of both compounds (Table 1).

Next we tested for synergism between LY294002 and 1,25(OH)₂D₃ in *Pten*^{-/-} MPEC, which were isolated from 8-10 week old animals with targeted deletion of *Pten* in the prostate and are 1,25(OH)₂D₃-insensitive. Pre-treatment of *Pten*^{-/-} MPECs (clone 11) with LY294004 partially restored sensitivity to 1,25(OH)₂D₃-mediated antiproliferative effects and synergized to inhibit the growth of the cells (Fig. 1C, Table 1, and Fig S1C).

These data demonstrated that LY294002 which is a potent inhibitor of both PI3K and AKT can synergize with 1,25(OH)₂D₃ to inhibit prostate cancer cell proliferation. However, LY294002 has recently been reported to have many other off-target effects (30). Therefore, to validate synergism with AKT-inhibition specifically, we used two different AKT inhibitors which currently are in clinical trials for cancer treatment: API-2 (Triciribine), which selectively inhibits Akt1/2/3 without inhibiting PI3K or PDK (31) and GSK690693, an ATP competitive AKT inhibitor (32). We also expanded the effects to primary human prostate cancer epithelial cells.

The combination of 1,25(OH)₂D₃ with API-2 elicited synergistic growth inhibition of DU145 cells as well as of human primary prostate cancer cell strains WFU275CA and WFU273CA (Table 2, Table S1, Fig 2A,B Fig S2A,B, and Fig S3). The combination of another AKT inhibitor GSK690693 with 1,25(OH)₂D₃ also elicited strong to very strong synergistic growth inhibition of DU145 cells (Fig.2C, S2C and Table 2) and of human primary prostatic epithelial cells (Fig.2D and S2D). Together these data demonstrated that inhibition of the AKT pathway synergizes with 1,25(OH)₂D₃ to inhibit the growth of prostate cancer cell.

Having demonstrated synergism between $1,25(\text{OH})_2\text{D}_3$ and AKT inhibitors we sought to explore the mechanism. First, we performed video time-lapse microscopy analysis of DU145 cells treated with API-2 (0.25 μM) or $1,25(\text{OH})_2\text{D}_3$ (100 nM) alone as well as in combination. We did not observe any considerable apoptosis in any of the treatments. Next we evaluated cell cycle distribution of DU145 cells treated with API-2 or $1,25(\text{OH})_2\text{D}_3$. Cell cycle analysis demonstrated a small but statistically significant increase in G_1 -arrested cells treated with the combination of API-2 at 0.25 μM with $1,25(\text{OH})_2\text{D}_3$ at 10 nM compared to either agent alone (Fig. 3A).

Since we were unable to detect apoptosis and the effects on cell cycle, although significant, were modest, we next evaluated the effects of the combination on senescence. In the human primary cancer cell strain WFU273Ca $1,25(\text{OH})_2\text{D}_3$ was able to induce SA- β -Gal activity which was associated with morphological features consistent with senescence (Fig 3B,C). Treatment with 100 nM $1,25(\text{OH})_2\text{D}_3$ was able to induce senescence in 16.8% of the WFU273CA cells (Table S2). The combination of API-2 and $1,25(\text{OH})_2\text{D}_3$ cooperated with each other at the higher doses tested to induce greater SA- β -Gal activity which was statistically significant by ANOVA (Fig 3B, 3C and Table S2).

DU145 cells also showed significant cooperativity for induction of SA- β -gal. Treatment with 0.25 μM API-2 induced senescence in 5.11% of DU145 cells and 100 nM $1,25(\text{OH})_2\text{D}_3$ induced senescence in 2.64% of the cell; the combination of the two led to 10.48% of the cells to undergo senescence (Fig 3D) which was statistically significant by ANOVA. Representative photographs are shown in Fig. S4.

Together these data suggest that synergism between API-2 and $1,25(\text{OH})_2\text{D}_3$ in prostate cancer cells might be a result of cooperative induction of cell cycle arrest and senescence.

SOW TASK 2 progress:

Currently we are performing experiments described in the SOW Task 2A which aims to determine downstream mediator of the AKT axis and $1,25(\text{OH})_2\text{D}_3$ signaling that is involved in the synergism between AKT inhibitors and $1,25(\text{OH})_2\text{D}_3$. Our main hypothesis is that p21 and p27 are the primary players involved in this interplay. Thus, we are evaluating the levels of expression of p21 and p27 protein in sub-cellular compartments of prostate cells upon treatment with $1,25(\text{OH})_2\text{D}_3$ alone, AKT inhibitor alone or the combination of these two agent. We are utilizing Immunofluorescence microscopy methods as well as Immunoblotting of the cytoplasmic and nuclear fractions of the cells.

We are in the process of the establishing the shRNA vectors that will knock down p21 and p27 protein in order to evaluate the importance of these proteins in the cross talk between the AKT signaling and antiproliferative $1,25(\text{OH})_2\text{D}_3$ -signlaing (as described on the SOW Task 2B).

Key research accomplishments

- a manuscript in preparation for submission
- have generated the MPEC clones with Pten knocked down with shRNA as well as control clones (with confirmed stable knockdown by Immunoblott)
- have generated Pten^{lox/lox} MPEC and Pten^{-/-} (Pten^{lox/lox} infected with Cre-recombinase lentivirus) cell lineages (with confirmed Pten deletion by PCR and Immunoblot)

Reportable outcomes

We have demonstrated that pharmacological inhibitors of PI3K or Akt restored sensitivity to antiproliferative effects of 1,25(OH)₂D₃ in 1,25(OH)₂D₃-insensitive cells and synergized to inhibit the growth of Pten^{-/-} mouse prostatic epithelial cells, DU145, LNCaP and primary human prostate cancer cell strains. The inhibitors used included API-2 (Triciribine) and GSK690693 which are currently in clinical trials for treatment of cancer. The combination of 1,25(OH)₂D₃ and AKT inhibitor API-2 led to a cooperative increase in G₁ arrest and in the induction of senescence. AKT is commonly activated by PTEN loss, therefore we evaluated the role of Pten in responsiveness to 1,25(OH)₂D₃ using shRNA knockdown and by *in vitro* knockout of Pten. We found that Pten status did not affect responsiveness to the antiproliferative action of 1,25(OH)₂D₃.

Conclusions

We found that Pten status *per se* did not affect responsiveness to the antiproliferative action of 1,25(OH)₂D₃. In addition, we discovered that pharmacological inhibitors of PI3K or AKT are capable of restoring sensitivity to antiproliferative effects of 1,25(OH)₂D₃ in 1,25(OH)₂D₃-insensitive cells. In addition, the PI3K/AKT inhibitors tested synergized to inhibit the growth of Pten^{-/-} mouse prostatic epithelial cells, DU145, LNCaP and primary human prostate cancer cell strains. Inhibitors of the PI3K/AKT pathway included Triciribine and GSK690693 which are currently in clinical trials for treatment of cancer and the effect of the combination was 10 to 1000 times greater than the AKT inhibitor alone as was demonstrated by Dose Reduction Indexes. This synergistic effect has not been previously demonstrated and could significantly enhance the therapeutic efficacy of these clinically relevant drugs while reducing the potential side effects. Thus, these findings provide the rationale for the development of therapeutic interventions utilizing 1,25(OH)₂D₃ or its analogs combined with inhibition of PI3K/AKT for the treatment of prostate cancer.

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1,25-dihydroxyvitamin D₃ and PI3K/AKT inhibitors synergistically inhibit growth and cooperatively induce cell cycle arrest and senescence in prostate cancer cells

Linara S. Axanova¹, Yong Q. Chen¹, Thomas McCoy², Guangchao Sui¹ and Scott D. Cramer^{1,2}.

¹*Cancer Biology and* ²*Public Health Science Departments, Wake Forest University School of Medicine, Winston Salem, NC.*

Correspondence: Scott D. Cramer, Department of Cancer Biology, Wake Forest University School of Medicine, Medical Center Blvd. Winston-Salem, NC 27157. Phone: (336) 713-7651, FAX (336) 713-7661, E-Mail scramer@wfubmc.edu

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Statement of Translational Relevance

The presented manuscript demonstrates that various pharmacological inhibitors of the PI3K/AKT pathway (including Triciribine and GSK690693 which are currently in clinical trials for treatment of cancer) synergized with 1-alpha, 25-dihydroxy vitamin D₃ (1,25(OH)₂D₃) to inhibit the growth of prostate cancer cells. This effect was demonstrated in multiple prostate cancer cell types and strains. The effect of the combination was 10 to 1000 times greater than the AKT inhibitor alone as was demonstrated by Dose Reduction Indexes. This synergistic effect has not been previously demonstrated and could significantly enhance the therapeutic efficacy of these clinically relevant drugs while reducing the potential side effects.

Abstract: Purpose: 1- α , 25-dihydroxy vitamin D₃ (1,25(OH)₂D₃) inhibits proliferation in multiple cancer cell types including prostate cells and upregulates p21 and/or p27, while loss of Pten and PI3K/AKT activation stimulates survival and downregulates p21 and p27. We hypothesized that inhibition of the PI3K/AKT pathway synergizes with the antiproliferative signaling of 1,25(OH)₂D₃. Experimental Design: Viability, cell cycle distribution and senescence of cells were evaluated upon combinational treatment with 1,25(OH)₂D₃ and pharmacological PI3K/AKT inhibitors. Pten was knockdown by shRNA or knockout by Cre-recombinase and responsiveness to 1,25(OH)₂D₃ was evaluated. Results: Pharmacological inhibitors of PI3K or Akt restored sensitivity to antiproliferative effects of 1,25(OH)₂D₃ in 1,25(OH)₂D₃-insensitive cells and synergized to inhibit the growth of Pten^{-/-} mouse prostatic epithelial cells, DU145, LNCaP and primary human prostate cancer cell strains. The inhibitors used included API-2 (Triciribine) and GSK690693 which are currently in clinical trials for treatment of cancer. The combination of 1,25(OH)₂D₃ and AKT inhibitor API-2 led to a cooperative increase in G₁ arrest and in the induction of senescence. As AKT is commonly activated by PTEN loss, we evaluated the role of Pten in responsiveness to 1,25(OH)₂D₃. We found that Pten knockdown or knockout did not reduce responsiveness to 1,25(OH)₂D₃.

Conclusions: These findings provide the rationale for the development of therapeutic interventions utilizing 1,25(OH)₂D₃ or its analogs combined with inhibition of PI3K/AKT for the treatment of prostate cancer.

Introduction

Prostate cancer (CaP) is the most commonly diagnosed cancer and the second most common cause of cancer death in American men (1). It has been suggested that the development of clinical prostate cancer may be associated with vitamin D₃ deficiency (2, 3). Vitamin D is a hormone that can be obtained from the diet or produced endogenously by a series of reactions that culminates in the most active metabolite of vitamin D, 1 α , 25(OH)₂-vitamin D₃ (1,25(OH)₂D₃). 1,25(OH)₂D₃ elicits antiproliferative effects in a variety of cancer cell types including cell lines derived from prostate (4, 5). The ability of 1,25(OH)₂D₃ to inhibit prostate growth was also demonstrated in primary prostatic cells from histologically normal, benign prostatic hyperplasia, and prostate cancer specimens (6), in multiple prostate cancer cell lines (7-9), in xenograft models of prostate cancer (10, 11) as well as in Dunning rat prostate model (12). The anticancer mechanisms of 1,25(OH)₂D₃'s action include induction of cell cycle arrest, promotion of differentiation, inhibition of proliferation and angiogenesis, as well as inhibition of invasive and migratory potential of cancer cells [reviewed in (13)].

Classical actions of 1,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR) which is a member of a superfamily of nuclear steroid hormone receptors. Upon 1,25(OH)₂D₃ binding VDR translocates to the nucleus, dimerizes with retinoid X receptor (RXR) and modulates the expression of target genes. Although a number of 1,25(OH)₂D₃ responsive genes are known, the exact mechanism of growth regulation by 1,25(OH)₂D₃ is not completely defined; however, an increase in p21 and/or p27 is an almost universal feature (4).

One of the central contributing factors which facilitates the survival of prostate cancer cells is attributed to the phosphoinositol-3 kinase PI3K-AKT pathway. Activated AKT phosphorylates a host of proteins that affect cell growth, cell cycle entry, and cell survival. The AKT pathway

presents an attractive target for anticancer therapies and several AKT inhibitors have been developed that demonstrate anticancer activity in preclinical and clinical studies (14). In prostate cancer, activation of AKT occurs most frequently due to the loss of tumor suppressor phosphatase and a tensin homologue deleted in chromosome ten (PTEN)(15-17). Loss of PTEN protein occurs in 20% of primary prostate tumors and this loss is highly correlated with advanced tumor grade and stage with 50% of metastatic tumors exhibiting a loss of PTEN protein (18). Moreover, loss of heterozygosity (LOH) is found in 20% to 60% of metastatic tumors (19). Data suggests that advancing disease is associated with a progressive loss of PTEN or an accumulation of mutations in the PTEN gene. Loss of PTEN and activation of AKT has been shown to downregulate the expression of p21 and p27 by a number of mechanisms (20-25). Since the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ involve upregulation of p21 and/or p27 (4) while activation of PI3K/AKT downregulates their expression (25), we hypothesized that pharmacological inhibitors of AKT will cooperate with the antiproliferative actions of $1,25(\text{OH})_2\text{D}_3$ in prostate cancer cells.

Our results demonstrate that inhibition of PI3K or AKT synergized with $1,25(\text{OH})_2\text{D}_3$ to inhibit the growth of human prostate cancer cell lines, primary human prostate cancer strains, and mouse prostatic epithelial cells (MPEC) and led to cooperative induction of G_1 arrest and senescence. Responsiveness to the antiproliferative effects of $1,25(\text{OH})_2\text{D}_3$ was not dependent on Pten status. These findings provide the rationale for prostate cancer therapies involving use of AKT inhibitors and $1,25(\text{OH})_2\text{D}_3$ in adjunctive therapy.

Materials and Methods

Materials. 1,25(OH)₂D₃ (Biomol, Plymouth Meeting, PA) was reconstituted in 100% ethanol and stored at -80°C. LY294002 (Sigma-Aldrich Co., St Louis, MO), GSK690693 (26) (a generous gift from GlaxoSmithKline, Collegeville, PA) and API-2 (27) (Calbiochem, La Jolla, CA) were reconstituted in DMSO and stored at -20°C.

MPEC with *in vivo* deletion of Pten: Pten^{-/-} MPEC were isolated from anterior prostates of 8-10 week old Pten^{lox/lox} mice expressing Cre recombinase under the control of a modified rat prostate-specific probasin promoter (pbCre) (28). MPECs wild type for Pten were isolated from anterior prostates of 8-10 week old Pten^{wt/lox; pbCre⁻} mice. The method of isolation was developed in our laboratory and is previously described (29). MPEC were clonally selected using serial dilution as described (30) and Pten status was confirmed by PCR and Immunoblot (See Supplemental Methods).

MPECs with *in vitro* deletion of Pten: Pten^{lox/lox} anterior MPECs were isolated from 8 week old Pten^{lox/lox; pbCre⁻} animals as described (29) and infected with self-deleting Cre-recombinase lentivirus (31). Deletion was validated by PCR and immunoblot (See Supplemental Methods).

Tissue culture. LNCaP and DU145 cells (both from American Type Culture Collection, Manassas, VA) were grown in RPMI-1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin. MPEC were grown as described previously (29). Two separate human prostate epithelial cancer cell strains were obtained from separate patients (labeled WFU273Ca and WFU275Ca), validated for histological origin, and maintained as previously described (32).

shRNA Infection. WFU3 MPEC (29) were infected with lentivirus expressing shRNA targeting Pten (gaa cct gat cat tat aga tat t) or control shRNA (gggc cat ggc acg tac ggc aag). Lentivirus production and infection procedure were previously described (33). MPECs were clonally selected using serial dilution and Pten status was confirmed by Immunoblot.

Growth assays for synergism determination. Cells were plated at 10^4 cells per 35 mm dish in triplicate. To determine synergism cells were treated with increasing doses of AKT inhibitor, increasing doses of $1,25(\text{OH})_2\text{D}_3$ or multiple combinations of AKT inhibitor and $1,25(\text{OH})_2\text{D}_3$. Briefly, 48 hr after plating the cell growth medium was replaced with 1 ml of experimental medium containing twice (2x) the indicated concentration of a PI3K/AKT inhibitor or vehicle (DMSO, 1x = 0.1% V/V). One hour later 1 ml of medium containing twice the final concentration of $1,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol, 1x = 0.1% V/V) was added to each dish. DU145, LNCaP and Pten^{-/-} MPEC remained in the experimental medium until the controls reached 80-90% confluence. For WFU273Ca and WFU275Ca and for all experiments using GSK690693 the experimental medium was replaced every 48hr. Viable cells were counted with a hemacytometer after trypan blue exclusion. Results of representative experiments are shown.

Flow cytometry: Flow cytometry was performed as described (5) using Becton Dickinson FACSCaliber and analyzed by the Cell Quest Pro v.6.0 program (Becton Dickinson, Mansfield, MA). Data were processed with ModFit LT v.2.0 software (Verity Software House, Topsham, MN). Each treatment was performed in triplicate and the experiment was conducted three times.

Senescence-associated (SA)- β -galactosidase (gal) activity: Cells were cultured and treated as described above. After 5-7 days of treatment (SA)- β -galactosidase activity was evaluated by the method of Dimri et al. (34). Digital images were taken from 10 random areas at 20X magnification. Digital images were evaluated in Photoshop CS2 9.0.2 (Adobe Systems, San

Jose, CA). The number of SA- β -gal positive cells was counted in each image and presented as percent of total cell number \pm SE.

Statistical Analyses: Synergism was assessed with CalcuSyn software (Biosoft, Ferguson, MO) as previously described (5). Briefly, the dose effect for each drug alone was determined based on the experimental observations using the median effect principle: the combination index (CI) for each combination was calculated according to the following equation: $CI = [(D)_1/(D_x)_1] + [(D)_2/(D_x)_2] + [(D)_1(D)_2/(D_x)_1(D_x)_2]$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. CI = 1 represents the conservation isobologram and indicates additive effects. CI values < 1 indicate a more than expected additive effect (synergism). All other statistical analyses were performed using the statistical software package NCSS 2002 (Number Cruncher Statistical Systems, Kaysville, UT). Differences in growth data were determined by two-way ANOVA controlling for 1,25(OH) $_2$ D $_3$ or PI3K/AKT inhibitor dose with post hoc analysis by Fisher's test. Cell cycle distribution and senescence analyses were performed using two-way ANOVA with post hoc analysis by Fisher's LSD test. In all cases, $P < 0.05$ was considered significant.

Results

1,25(OH)₂D₃ and PI3K/Akt inhibitors synergistically inhibit prostate cancer cell growth.

To test whether inhibition of the PI3K/Akt pathway synergizes with 1,25(OH)₂D₃ treatment to inhibit the growth of prostatic cells we first utilized a pharmacological inhibitor of PI3K/AKT, LY294002 (35). Cells were treated with increasing doses of 1,25(OH)₂D₃ or LY294002 or with multiple combinations of the two compounds and the number of viable cells was assessed. Synergism was assessed with the Chou-Talalay method (36) in which the combination index (CI) values < 1 indicates presence of synergism.

We found that LNCaP cells, which lack functional PTEN and are moderately sensitive to growth inhibition by 1,25(OH)₂D₃, demonstrated statistically significant synergism between 1,25(OH)₂D₃ and LY294002 at lower doses of 1,25(OH)₂D₃ (Fig. 1A, Table 1, and Fig S1A). The combination of 1 nM 1,25(OH)₂D₃ treatment with 0.2 μ M, 1 μ M or 5 μ M LY294002 demonstrated statistically significant synergism as determined by CI values from isobologram analysis (Table 1). The dose-reduction index (DRI) for each of the drugs is also depicted in Table 1 and presents a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone (36).

DU145 cells (wild-type PTEN) are known to be resistant to the antiproliferative effects of 1,25(OH)₂D₃ (37). Consistent with this, we found that 1,25(OH)₂D₃ did not significantly affect growth of DU145 cells (Fig 1B and S1B). While 100 nM 1,25(OH)₂D₃ did not inhibit growth of DU145 cells, pretreatment of DU145 cells with 5 μ M LY294002 sensitized the cells to 1,25(OH)₂D₃ treatment leading to a reduction in the number of viable cells to 40% compared to treatment with LY294002 alone (Fig 1B and S1B). We found significant synergism across multiple doses of both compounds (Table 1).

Next we tested for synergism between LY294002 and 1,25(OH)₂D₃ in *Pten*^{-/-} MPEC, which were isolated from 8-10 week old animals with targeted deletion of *Pten* in the prostate and are 1,25(OH)₂D₃-insensitive. Pre-treatment of *Pten*^{-/-} MPECs (clone 11) with LY294004 partially restored sensitivity to 1,25(OH)₂D₃-mediated antiproliferative effects and synergized to inhibit the growth of the cells (Fig. 1C, Table 1, and Fig S1C).

These data demonstrated that LY294002 which is a potent inhibitor of both PI3K and AKT can synergize with 1,25(OH)₂D₃ to inhibit prostate cancer cell proliferation. However, LY294002 has recently been reported to have many other off-target effects (38). Therefore, to validate synergism with AKT-inhibition specifically, we used two different AKT inhibitors which currently are in clinical trials for cancer treatment: API-2 (Triciribine), which selectively inhibits Akt1/2/3 without inhibiting PI3K or PDK (27) and GSK690693, an ATP competitive AKT inhibitor (26). We also expanded the effects to primary human prostate cancer epithelial cells.

The combination of 1,25(OH)₂D₃ with API-2 elicited synergistic growth inhibition of DU145 cells as well as of human primary prostate cancer cell strains WFU275CA and WFU273CA (Table 2, Table S1, Fig 2A,B Fig S2A,B, and Fig S3). DU145 cells treated with the combination of API-2 and 1,25(OH)₂D₃ demonstrated strong synergism at all doses tested as determined by CI values from isolobolgrams (Table 2). Note that the treatment with API-2 and 1,25(OH)₂D₃ was applied only once after which the cells were allowed to grow until the cells in either of the treatment groups were 90% confluent.

The combination of API-2 and 1,25(OH)₂D₃ used for treatment of primary epithelial prostate cancer cell strain WFU275Ca demonstrated statistically significant moderate to strong synergism (Table 2) with DRI values as high as 283.0 for 1,25(OH)₂D₃ and 9.6 for API-2. Similar results were observed in WFU273Ca (Fig.S3A and Table S1).

The combination of another AKT inhibitor GSK690693 with $1,25(\text{OH})_2\text{D}_3$ also elicited strong to very strong synergistic growth inhibition of DU145 cells (Fig.2C, S2C and Table 2). DRI values for GSK690693 ranged from 3.5 to 214.4 for GSK690693. DRI is calculated using the equation: $(\text{DRI})_1 = (D_x)_1 / D_1$ and $(\text{DRI})_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. DRI values for treatment of DU145 with $1,25(\text{OH})_2\text{D}_3$ are extremely high as the x effect of the $1,25(\text{OH})_2\text{D}_3$ alone was near zero.

Treatment of cells with and $1,25(\text{OH})_2\text{D}_3$ were applied every 48hr because single treatment with GSK690693 and $1,25(\text{OH})_2\text{D}_3$ did not render a synergistic effect (data not shown). We also tested the combination of GSK690693 with $1,25(\text{OH})_2\text{D}_3$ in human primary prostatic epithelial cells (Fig.2D and S2D) which elicited strong synergism and DRI values as high as 5158.7 for $1,25(\text{OH})_2\text{D}_3$ and as high as 51.7 for GSK690693 (Table 2). Together these data support our hypothesis that inhibition of the AKT pathway synergizes with $1,25(\text{OH})_2\text{D}_3$ to inhibit the growth of prostate cancer cell.

$1,25(\text{OH})_2\text{D}_3$ and PI3K/Akt inhibitors cooperate to inhibit cell cycle progression and induce senescence

Having demonstrated synergism between $1,25(\text{OH})_2\text{D}_3$ and AKT inhibitors we sought to explore the mechanism. First, we performed video time-lapse microscopy analysis of DU145 cells treated with API-2 (0.25 μM) or $1,25(\text{OH})_2\text{D}_3$ (100 nM) alone as well as in combination. We did not observe any considerable apoptosis in any of the treatments (data not shown). Next we evaluated cell cycle distribution of DU145 cells treated with API-2 or $1,25(\text{OH})_2\text{D}_3$. Cell cycle analysis demonstrated a small but statistically significant increase in G_1 -arrested cells treated

with the combination of API-2 at 0.25 μ M with 1,25(OH) $_2$ D $_3$ at 10 nM compared to either agent alone (Fig. 3A).

Since we were unable to detect apoptosis and the effects on cell cycle, although significant, were modest, we next evaluated the effects of the combination on senescence. In the human primary cancer cell strain WFU273Ca 1,25(OH) $_2$ D $_3$ was able to induce SA- β -Gal activity which was associated with morphological features consistent with senescence (Fig 3B,C). Treatment with 100 nM 1,25(OH) $_2$ D $_3$ was able to induce senescence in 16.8% of the WFU273CA cells (Table S2). The combination of API-2 and 1,25(OH) $_2$ D $_3$ cooperated with each other at the higher doses tested to induce greater SA- β -Gal activity which was statistically significant by ANOVA (Fig 3B, 3C and Table S2). Interestingly, inhibition of the AKT pathway with API-2 alone induced senescence in WFU273Ca cells with 0.5 μ M API-2 causing 30% of the cells to undergo senescence, suggesting a role of the AKT pathway in the prevention of senescence in these cells (Table S2).

DU145 cells also showed significant cooperativity for induction of SA- β -gal. Treatment with 0.25 μ M API-2 induced senescence in 5.11% of DU145 cells and 100 nM 1,25(OH) $_2$ D $_3$ induced senescence in 2.64% of the cell; the combination of the two led to 10.48% of the cells to undergo senescence (Fig 3D) which was statistically significant by ANOVA. Representative photographs are shown in Fig. S4.

Together these data suggest that synergism between API-2 and 1,25(OH) $_2$ D $_3$ in prostate cancer cells might be a result of cooperative induction of cell cycle arrest and senescence.

The role of Pten status in sensitivity to antiproliferative action of 1,25(OH) $_2$ D $_3$.

Activation of AKT in prostate cancer occurs most frequently due to the loss of the tumor suppressor phosphatase and a tensin homologue deleted in chromosome ten (PTEN), which is

found in 20% of primary tumors and more than 50% of metastatic tumors (18). Thus, we wanted to evaluate the role of Pten in the antiproliferative signaling of 1,25(OH)₂D₃. To test this we used Pten^{+/+} MPEC and Pten^{-/-} MPEC which were isolated from Pten^{wt/lox; pbCre⁻} and from Pten^{lox/lox; pbCre⁺} animals, respectively. Animals were 8-10 weeks old and while Pten^{wt/lox; pbCre⁻} prostates did not show any abnormalities. Pten^{-/-} prostates were presented with tumors consistent with other reports for this strain (39). Both types of MPEC were clonally selected using serial dilution as described (30) and Pten status was confirmed by PCR and Immunoblot (not shown).

Pten^{+/+} and Pten^{-/-} MPEC clones were treated with increasing doses of 1,25(OH)₂D₃ and while Pten^{+/+} clones showed a dose-dependent growth inhibition, the same concentrations of 1,25(OH)₂D₃ did not inhibit growth of Pten^{-/-} clones and in some clones marginally stimulated growth (Fig. 4A). This suggested that Pten loss might desensitize MPEC to the growth inhibitory effects of 1,25(OH)₂D₃; alternatively, other events associated with tumorigenesis resulting from Pten loss may be responsible for the observed insensitivity to 1,25(OH)₂D₃ antiproliferative action. Noteworthy, this resistance to the antiproliferative effects of 1,25(OH)₂D₃ in Pten^{-/-} MPEC was overcome by LY294002 treatment (Fig. 1C).

In order to evaluate the role of Pten in 1,25(OH)₂D₃-mediated growth inhibition in more controlled settings we generated MPEC in which Pten expression was suppressed using an shRNA approach. MPEC WFU3 (29) were infected with either lentivirus expressing scrambled shRNA or with lentivirus expressing shRNA targeting Pten. Single cell clones of each of the cell types were established and Pten status was verified by Immunoblot (Fig. S5A). WFU3 control MPEC clones and WFU3 MPEC clones with Pten knocked down to undetectable levels were treated with increasing doses of 1,25(OH)₂D₃. There was no significant difference in the response to 1,25(OH)₂D₃ based on Pten status; both cell types remained 1,25(OH)₂D₃-sensitive

(Fig. 4B). This suggested that suppression of Pten expression *per se* is not sufficient to abrogate the growth-inhibitory qualities of 1,25(OH)₂D₃ in MPEC.

Since gene suppression using an shRNA approach has the potential disadvantages of incomplete suppression of target gene expression and also possible off-target effects, we sought to establish a cell line with acute *in vitro* deletion of Pten. Thus, we isolated Pten^{lox/lox} MPEC from prostates of Pten^{lox/lox} Cre-recombinase negative animals and infected them with lentivirus expressing self-deleting Cre-recombinase (31). PCR and Immunoblot analysis of the deletion demonstrated the absence of Pten expression in the cells infected with Cre-recombinase (Fig. S5B and S5C).

The control Pten^{lox/lox} MPEC and Pten^{-/-} (Pten^{lox/lox} infected with Cre-recombinase lentivirus) cells were treated with increasing concentrations of 1,25(OH)₂D₃. We observed that *in vitro* loss of Pten did not significantly affect the ability of 1,25(OH)₂D₃ to inhibit the growth of the cells (Fig. 4C). These results were confirmed by clonogenic assays (Fig. S6, S7). Together, these data demonstrate that while *in vivo* loss of Pten was associated with resistance to 1,25(OH)₂D₃-mediated growth inhibition of MPEC, *in vitro* suppression or loss of Pten in MPECs was not associated with increased resistance to 1,25(OH)₂D₃.

Discussion

In this study we showed that AKT inhibitors in combination with 1,25(OH)₂D₃ synergistically inhibit the growth of prostate cancer cells. The effect was observed with multiple inhibitors of PI3K and/or AKT and in multiple cell lines as well as primary human prostate cancer samples. Interestingly both cells that were wild-type for Pten and cells that lacked Pten were sensitive to the synergistic effects observed, suggesting broad applicability to prostate cancer.

Mechanistically, we observed moderate effects on cell cycle progression and larger effects on the induction of senescence. No evidence of apoptosis was identified. These studies are important because the two AKT inhibitors that we used, API-2 (Triciribine) and GSK690693, have entered Phase I and II clinical trials for various cancers (14, 26, 40), and therefore the results could have clinical translation relatively rapidly. However, a factor complicating the use of AKT inhibitors is that the AKT pathway presents one of the most important pathways for normal cell survival. It is not clear yet whether treatment with AKT inhibitors will demonstrate acceptable levels of toxicity at doses that are therapeutically effective. Our data using $1,25(\text{OH})_2\text{D}_3$ in combination with the AKT inhibitors that show DRIs for the AKT drugs that range from 10 to over 1000, suggest that more therapeutic efficacy can be achieved by potentially less systemically toxic levels of the drugs.

$1,25(\text{OH})_2\text{D}_3$ used as a single agent has been shown to possess anticancer qualities in a number of cancer models but has toxicities associated with calcium mobilization at doses that are therapeutically effective. One strategy to overcome this problem is the creation of less calcemic $1,25(\text{OH})_2\text{D}_3$ analogs. Another strategy is to organize the treatment regimen in a way that allows reduction of side-effects; for instance, weekly oral high-dose $1,25(\text{OH})_2\text{D}_3$ administration is well-tolerated in androgen independent prostate cancer patients (41). Another strategy is to combine $1,25(\text{OH})_2\text{D}_3$ with other agents to develop therapeutic interventions that allow dose reduction, and thus alleviation of toxicities, while maintaining the growth inhibitory potential. Clinical trials utilizing $1,25(\text{OH})_2\text{D}_3$ or its analogs in combination with chemotherapy in advanced prostate cancer have demonstrated the feasibility of the use of $1,25(\text{OH})_2\text{D}_3$ for treatment of advanced prostate cancer (41-43). Beer et al. reported an 81% response rate for the combination of $1,25(\text{OH})_2\text{D}_3$ and docetaxel in metastatic prostate cancer versus an expected response of 40% to 50% for docetaxel alone (41). The DRIs for $1,25(\text{OH})_2\text{D}_3$ in combination

with AKT inhibitors, suggest that this combination could achieve therapeutic efficacy at non-toxic levels of $1,25(\text{OH})_2\text{D}_3$.

We investigated the mechanistic basis for the observed synergism and found that it was largely attributable to enhanced senescence. Interestingly, overexpression of activated AKT itself can lead to oncogene-induced senescence, which must be overcome for the development of invasive carcinoma. Expression of activated AKT-1 in the mouse ventral prostate (*AKT1*-tg mouse) leads to prostatic intraepithelial neoplasia (PIN), which by itself fails to progress to invasive carcinoma (44). These PIN lesions were shown to exhibit enhanced senescence with associated increased p27 expression (44). Human PIN lesions were also positive for increased senescence that was associated with higher p27 levels. The authors hypothesized that overexpression of activated AKT leads to senescence in a p27 dependent manner and this prevents the development of invasive carcinoma. To test this they crossed the *AKT1*-tg mouse with mice containing targeted disruption of p27 (44). Loss of a single copy of the p27 gene led to invasive carcinoma. These data indicate that critical levels of p27 expression are needed to act as an important brake on the promotion of invasive carcinoma by activation of the AKT pathway. The results suggest that agents that enhance p27 expression may be beneficial therapeutic agents for prostate cancer. $1,25(\text{OH})_2\text{D}_3$ increases the steady-state levels of p27 protein in prostate cancer cells (45) and our data show that it induces senescence in prostate cancer cells. These data suggest that $1,25(\text{OH})_2\text{D}_3$, or one of its analogs, should be explored for their ability to block progression of PIN lesions to invasive carcinoma.

In summary, $1,25(\text{OH})_2\text{D}_3$ and AKT inhibitors synergistically inhibit prostate cancer growth through induction of senescence. These data have implications for the clinical use of these agents in prostate cancer patients and may be important for progression in high-risk patients.

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Table 1) Synergism between 1,25(OH)₂D₃ and LY294002***

	1,25(OH) ₂ D ₃ , nM	LY294002, μM	CI*	DRI**		Synergism
				1,25(OH) ₂ D ₃	LY294002	
LNCaP						
	1	0.2	0.171	8.2	20.7	Strong
	1	1	0.298	10.9	4.8	Strong
	1	0.5	0.675	26.3	1.6	Moderate
DU145						
	1	2.5	0.408	513.8	2.5	Intermediate
	1	5	0.650	666.0	1.5	Intermediate
	10	2.5	0.382	58.2	2.8	Intermediate
	10	5	0.654	67.7	1.6	Intermediate
	10	10	0.801	117.6	1.3	Moderate
	100	2.5	0.489	6.4	3.0	Intermediate
	100	5	0.585	9.5	2.1	Intermediate
	100	10	0.701	14.9	1.6	Intermediate
Pten ^{-/-} MPEC						
	10	10	0.220	4.9*10 ⁴	4.6	Strong
	100	10	0.133	8969.7	7.5	Strong

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism)

**Dose Response Index

*** Data are from assays depicted in Figure S1A, only statistically significantly different means by ANOVA are depicted.

Table 2) Synergism between 1,25(OH)₂D₃ and AKT Inhibitors***

			DRI**		Synergism
1,25(OH)2D3, nM	AKT inhibitor, nM	CI*	1,25(OH)2D3	AKT inhibitor	
DU145					
AKT Inhibitor: API-2					
0.1	50	0.18*10 ⁻⁶	5.5*10 ⁴	4.0*10 ⁶	Very Strong
0.1	100	0.12*10 ⁻⁶	8.7*10 ⁴	4.4*10 ⁶	Very Strong
0.1	500	7.98*10 ⁻⁶	1.3*10 ⁵	1.9*10 ⁶	Very Strong
0.1	1000	9.82*10 ⁻⁶	1.2*10 ⁵	7.4*10 ⁵	Very Strong
1	50	0.75*10 ⁻⁶	1.3*10 ⁴	1.8*10 ⁷	Very Strong
1	100	0.55*10 ⁻⁶	1.8*10 ⁴	1.6*10 ⁷	Very Strong
1	500	0.60*10 ⁻⁶	1.7*10 ⁴	2.7*10 ⁶	Very Strong
1	1000	0.54*10 ⁻⁶	1.9*10 ⁴	1.6*10 ⁶	Very Strong
10	50	0.001	872.1	8.8*10 ⁶	Very Strong
10	100	0.001	1014.8	5.7*10 ⁶	Very Strong
10	500	0.001	1950.4	3.5*10 ⁶	Very Strong
10	1000	0.001	1461.5	1.1*10 ⁶	Very Strong
100	50	0.007	135.6	1.9*10 ⁷	Very Strong
100	100	0.005	206.7	1.9*10 ⁷	Very Strong
100	500	0.006	180.3	3.1*10 ⁶	Very Strong
100	1000	0.005	193.0	1.7*10 ⁶	Very Strong
WFU275CA					
AKT inhibitor: API-2					
0.1	0.1	0.799	283.0	1.3	Moderate
1	0.05	0.720	12.2	1.6	Moderate
1	0.1	0.714	36.9	1.5	Moderate
10	0.025	0.216	9.0	9.6	Strong
10	0.05	0.169	22.5	8.0	Strong
10	0.1	0.176	52.0	6.4	Strong
DU145					
AKT inhibitor: GSK690693					
10	1	0.003	1.1*10 ⁶	391.3	Very Strong
10	10	0.070	6.5*10 ⁴	14.3	Very Strong
100	0.1	0.0001	2.3*10 ⁴	2241.4	Very Strong
100	1	0.004	3.9*10 ⁴	268.7	Very Strong
100	10	0.010	1.5*10 ⁶	96.9	Very Strong
100	100	0.061	6.6*10 ⁶	16.3	Very Strong
100	1000	0.281	6.0*10 ⁷	3.5	Strong
WFU273CA					
AKT inhibitor: GSK690693					
0.1	1000	0.7730	5158.7	1.3	Moderate
1	10	0.062	37.1	28.5	Very Strong
1	100	0.120	255.9	8.7	Strong
1	1000	0.747	550.1	1.3	Moderate
10	10	0.115	10.5	51.7	Strong
10	100	0.234	14.1	6.1	Strong
10	1000	0.344	215.4	3.0	Intermediate

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism)

**Dose Response Index. DRI is calculated using the equation: $(DRI)_1 = (D_x)_1 / D_1$ and $(DRI)_2 = (D_x)_2 / D_2$, where $(D)_1$ and $(D)_2$ are the doses of drug that have x effect when used in combination and $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 that have the same x effect when used alone. DRI values for 1,25(OH)₂D₃ treatment in DU145 are high due to inability of the program to correctly calculate DRI based on the formula as the x effect of the 1,25(OH)₂D₃ alone is near zero.

*** Data are from assays depicted in Figure S2 and S3, only statistically significantly different means by ANOVA are depicted.

Figure 1

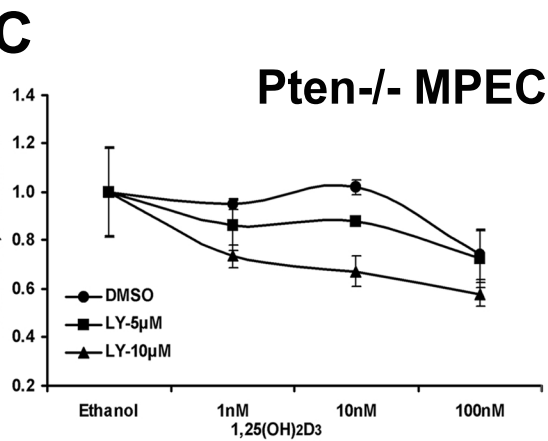
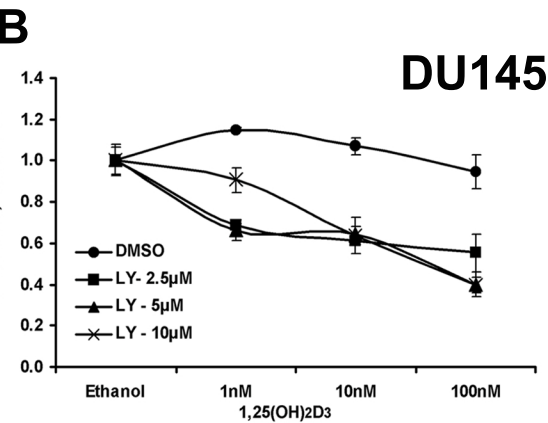
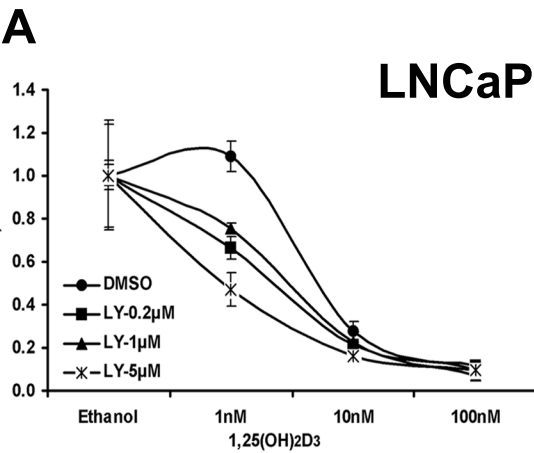
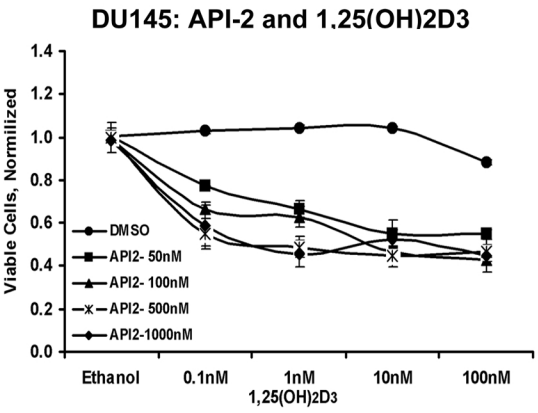
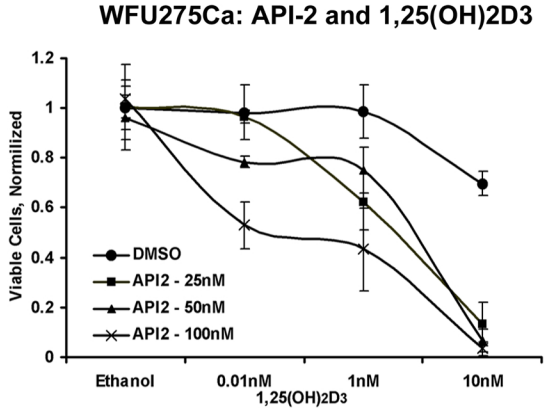


Figure 2

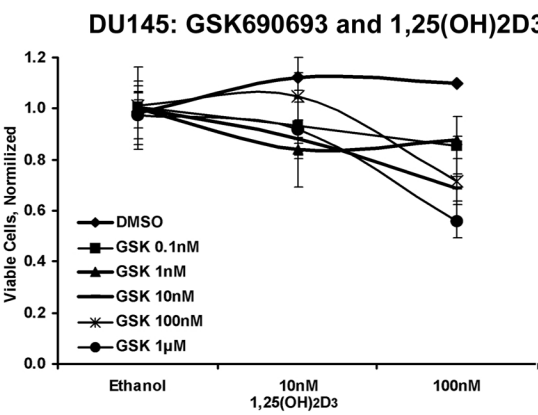
A



B



C



D

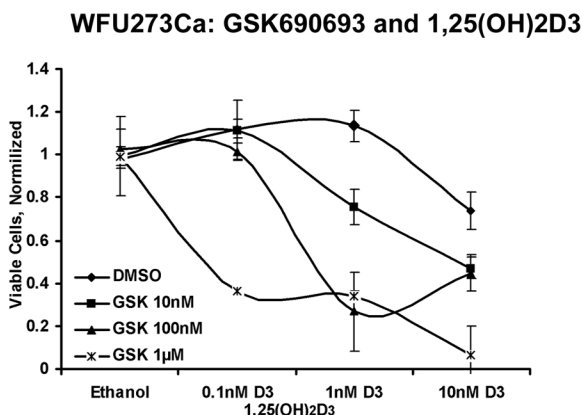
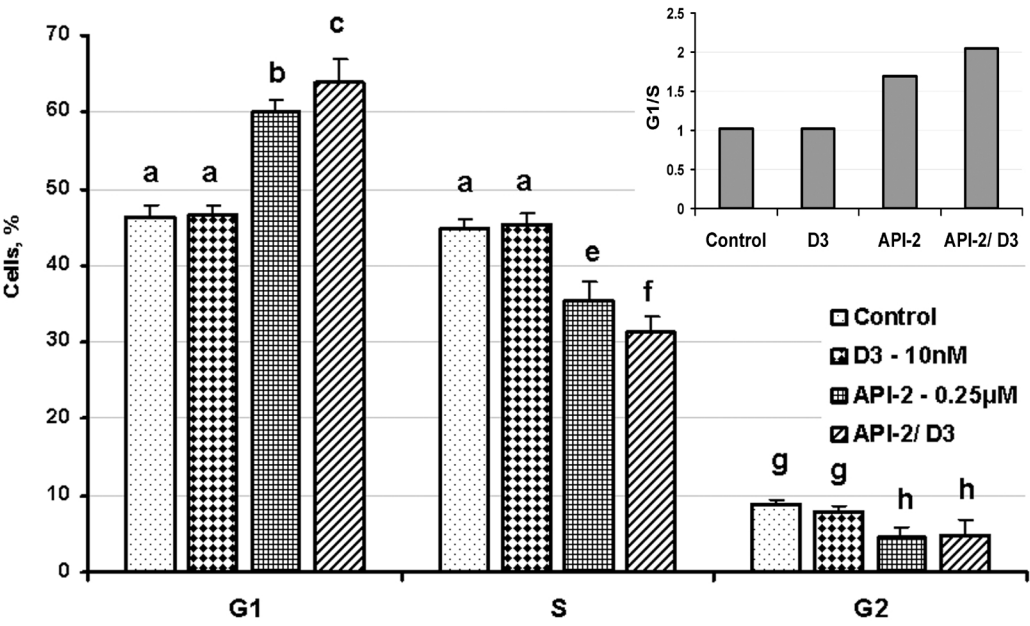
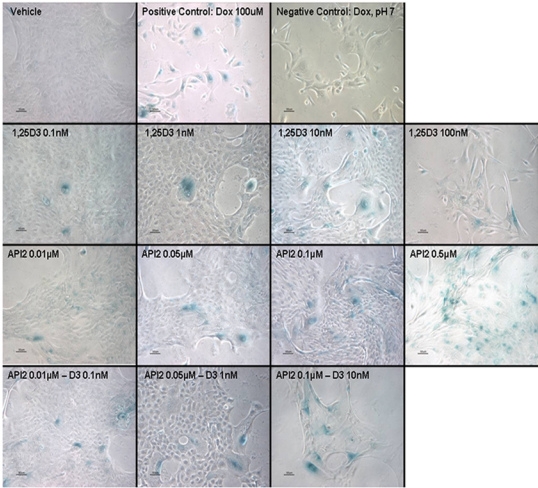


Figure 3

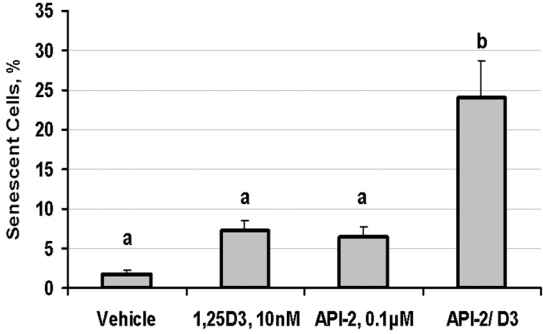
A



B



C



D

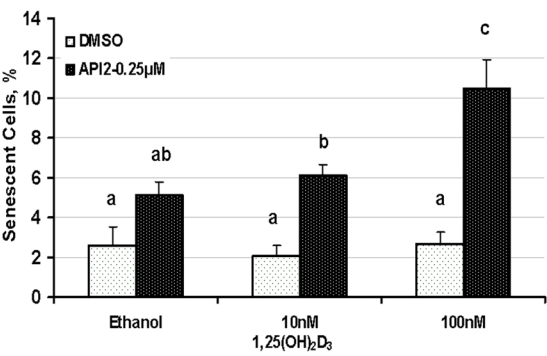
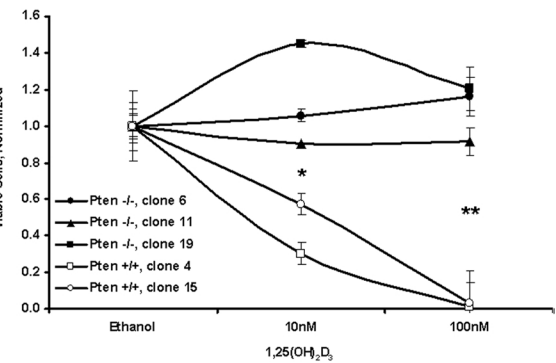
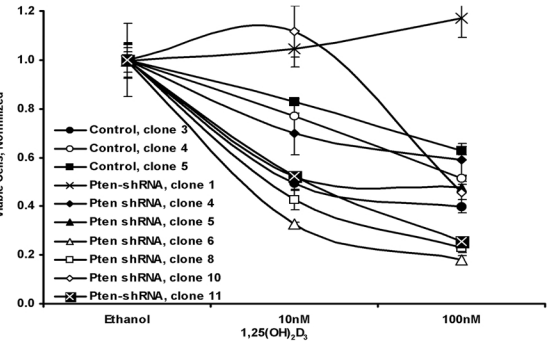


Figure 4

A



B



C

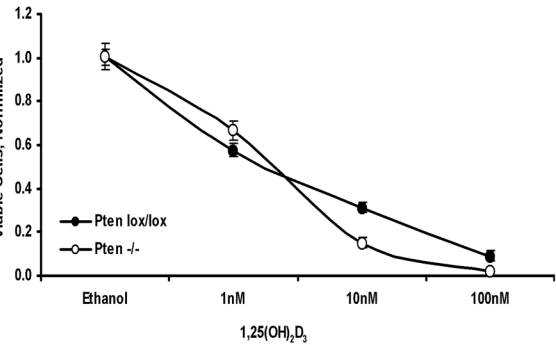


Figure Legends

Figure 1. LY294002 and 1,25(OH)₂D₃ synergistically inhibits growth of LNCaP, DU145 and Pten^{-/-} MPEC. Growth inhibition of LNCaP(**A**), DU145(**B**) and Pten^{-/-} MPEC (in vivo deletion of Pten) (**C**) in response to LY294002 and 1,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in Materials and Methods. Each point represents the mean and standard deviation of triplicate plates after normalization of cell number to controls (0.1% ethanol).

Figure 2. AKT inhibitors API-2 and GSK690693 synergize with 1,25(OH)₂D₃ to inhibit growth of DU145 cells and human primary prostate cancer strains.. Growth inhibition of DU145 cells (**A**) and human primary cancer cell strain WFU275CA (**B**) in response to API-2 and 1,25(OH)₂D₃ alone or in combination. Growth inhibition of DU145 cells (**C**) and human primary cancer cell strain WFU273CA (**D**) in response to GSK690693 and 1,25(OH)₂D₃ alone or in combination. Cells were grown, treated and analyzed as described in Materials and Methods.

Figure 3. AKT inhibitor API-2 and 1,25(OH)₂D₃ cooperate to induce G1-arrest and senescence. (**A**) 1,25(OH)₂D₃ and API-2 induce G1 arrest in cooperative manner in DU145 cells. DU145 cells were treated with the indicated doses for 24 hr and evaluated for cell cycle distribution as described in the Materials and Methods section. Insert demonstrates the G1/S ratios. Values are means for the triplicates ± SD. Means without a common letter are significantly different by ANOVA (*P* < 0.05). (**B**) 1,25(OH)₂D₃ and API-2 induce (SA)-β-galactosidase activity in a cooperative manner in WFU273CA cells. (**C**) Quantitative data from multiple images of WFU273Ca cells treated as indicated. Analysis was performed as described in the Materials and Methods section. Means ± SE are shown. Means without a common letter are significantly different by ANOVA (*P* < 0.05). (**D**) Quantitative data from ten images of DU145

cells treated as indicated. Analysis was performed as described in the Materials and Methods section. Means \pm SE are shown. Means without a common letter are significantly different by ANOVA ($P < 0.05$). Representative photographs are shown in Fig S4. Cells were grown, treated and (SA)- β -galactosidase activity was evaluated as described in Materials and Methods.

Figure 4. *In Vivo* loss of Pten is associated with loss of sensitivity to 1,25(OH)₂D₃ in MPECs while *in vitro* suppression or loss of Pten is not sufficient to antagonize 1,25(OH)₂D₃-mediated growth suppression.

(A) Growth inhibition of Pten^{+/+} and Pten^{-/-} MPECs (*in vivo* loss of Pten) in response to 1,25(OH)₂D₃. Means for the triplicates \pm SD of a representative experiment are demonstrated. Interaction between Pten status and 1,25(OH)₂D₃ dose was analyzed by two-way ANOVA ($p=.003$). * $p=0.004$; ** $p<0.001$. **(B)** Growth inhibition of WFU3 MPECs infected with Pten shRNA or scrambled shRNA in response to 1,25(OH)₂D₃. Values are means for the triplicates \pm SD. No statistically different response to 1,25(OH)₂D₃ based on Pten status was observed. **(C)** Growth inhibition of Pten^{lox/lox} and Pten^{-/-} MPECs in response to 1,25(OH)₂D₃. Pten^{lox/lox} MPECs isolated from prostates of Pten^{lox/lox} Cre-recombinase negative animals and infected with lentivirus expressing self-deleting Cre-recombinase to generate Pten^{-/-} MPEC. Values are means for the triplicates \pm SD. No statistically different response to 1,25(OH)₂D₃ based on genetic background was observed.

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Supplemental Table 1) Synergism between 1,25(OH)₂D₃ and API-2***

	1,25(OH) ₂ D ₃ , nM	API-2, μM	CI*	DRI**		Synergism
				1,25(OH) ₂ D ₃	API-2	
WFU273CA						
	1	0.1	0.442	11.1	2.8	Intermediate
	10	0.025	0.705	1.6	14.2	Intermediate
	10	0.05	0.324	4.1	12.8	Intermediate
	10	0.1	0.134	15.3	14.6	Strong

*Combination Index (CI<0.1 indicates Very Strong Synergism; 0.1÷0.3 Strong Synergism; 0.3÷0.7 Synergism; 0.70÷0.85 Moderate Synergism; 0.85÷0.90 Slight Synergism; 0.90÷1.10 Nearly Additive Synergism)

**Dose Response Index

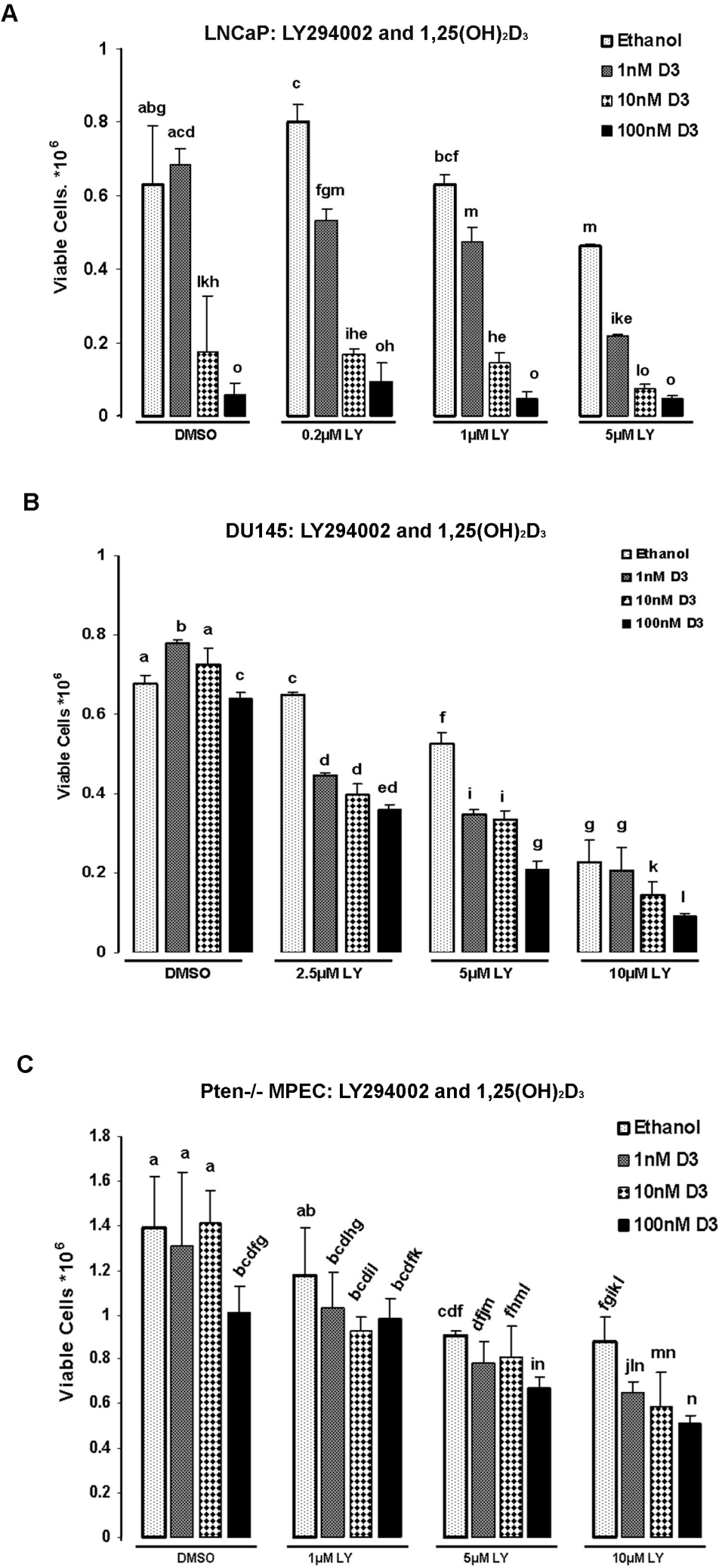
*** Data are from assays depicted in Figure S3A, only statistically significantly different means by ANOVA are depicted.

Supplemental Table 2 Induction of senescence in WFU273Ca primary prostate cancer cells*

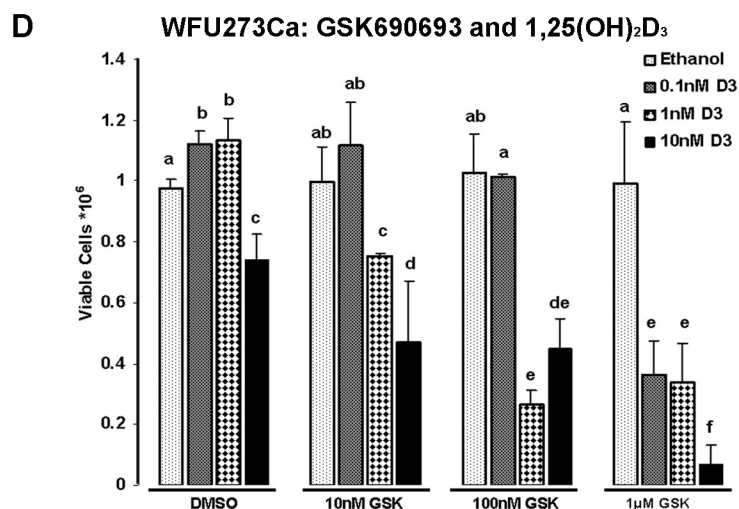
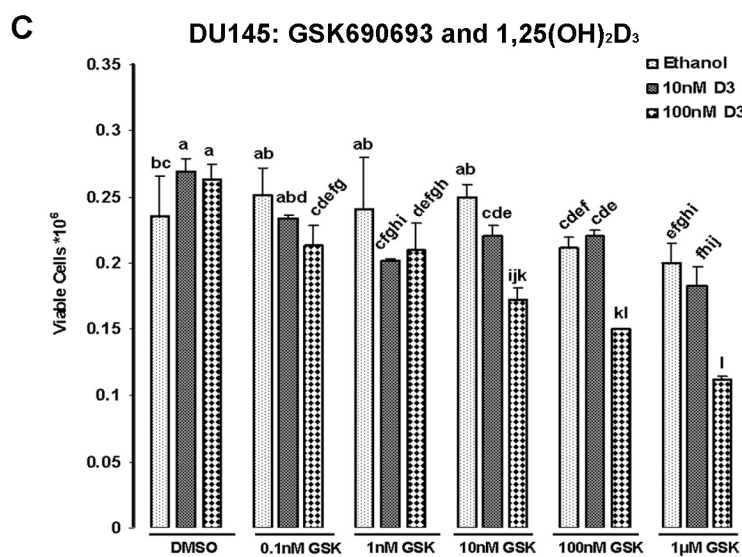
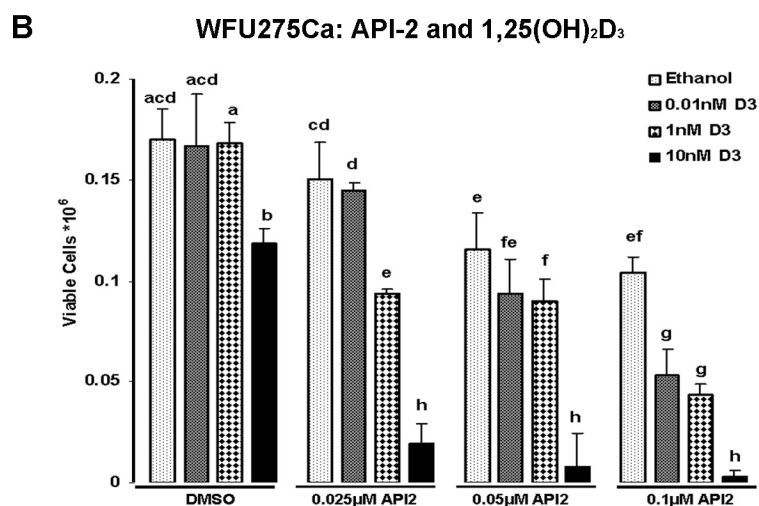
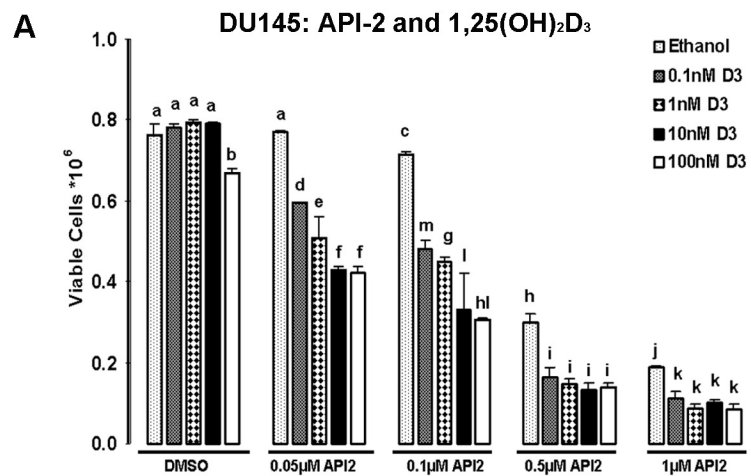
1,25D3 API-2	Vehicle	0.1nM	1nM	10nM	100nM
Vehicle	1.8(±0.4) ^a	2.8(±0.6) ^a	4.9(±2.8) ^a	7.3(±1.3) ^a	16.8(±2.3) ^b
0.01 µM	5.9(±1.1) ^a	3.5(±0.9) ^a			
0.05 µM	2.8(±0.5) ^a		3.8(±0.7) ^a		
0.1 µM	6.5(±1.2) ^a			24.0 (±5.6) ^c	
0.5 µM	30.0(±3.0) ^b				

*Percent of senescent cells as determined by (SA)-β-galactosidase activity. Means ± SE are shown. Means without a common letter are significantly different by ANOVA, $p < 0.05$.

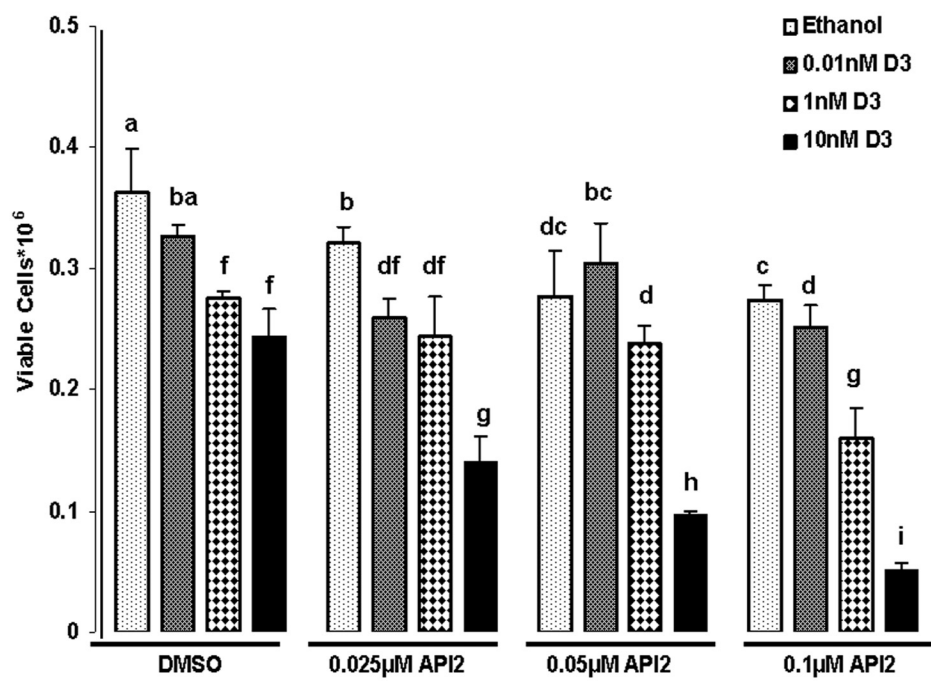
Supplemental Figure 1



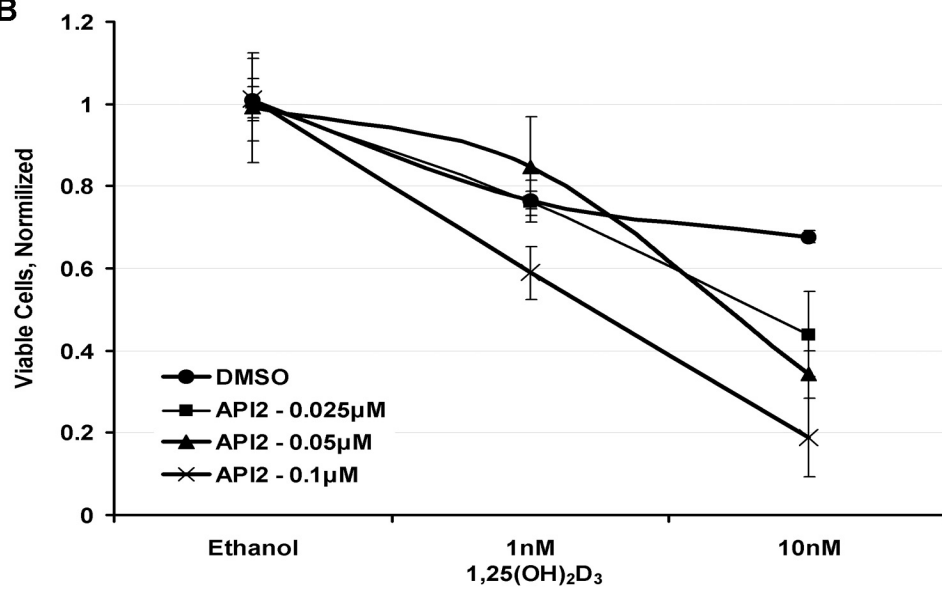
Supplemental Figure 2



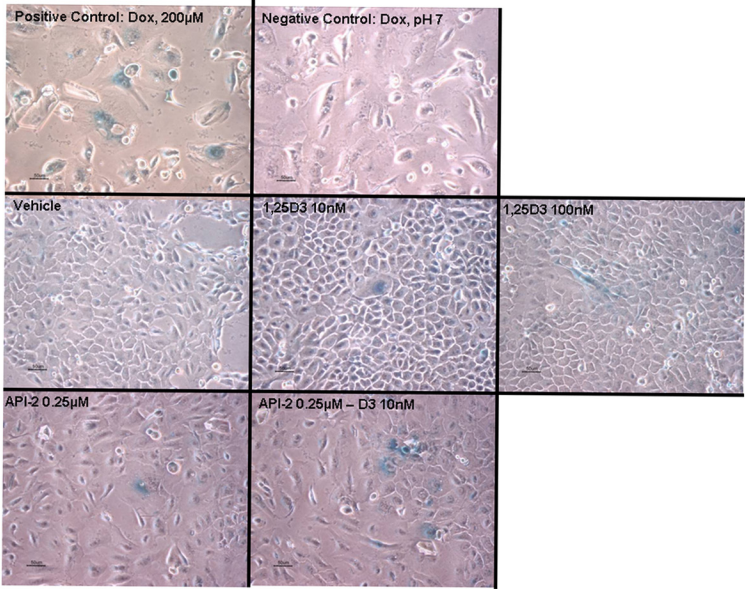
A



B

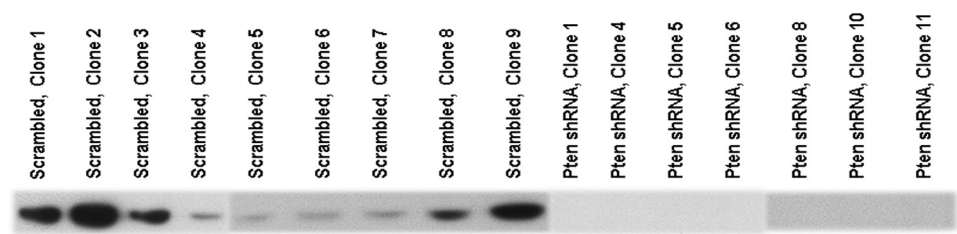


Supplemental Figure 4

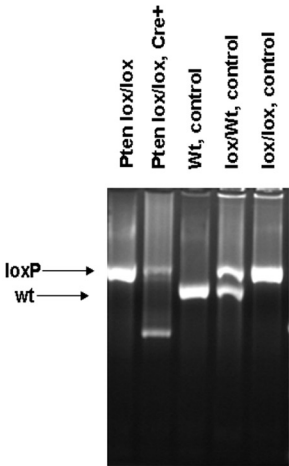


Supplemental Figure 5

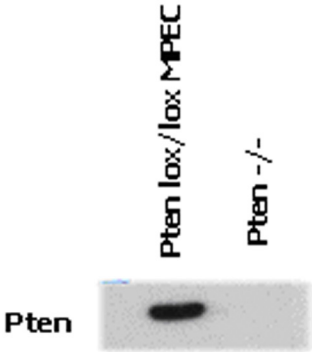
A



B

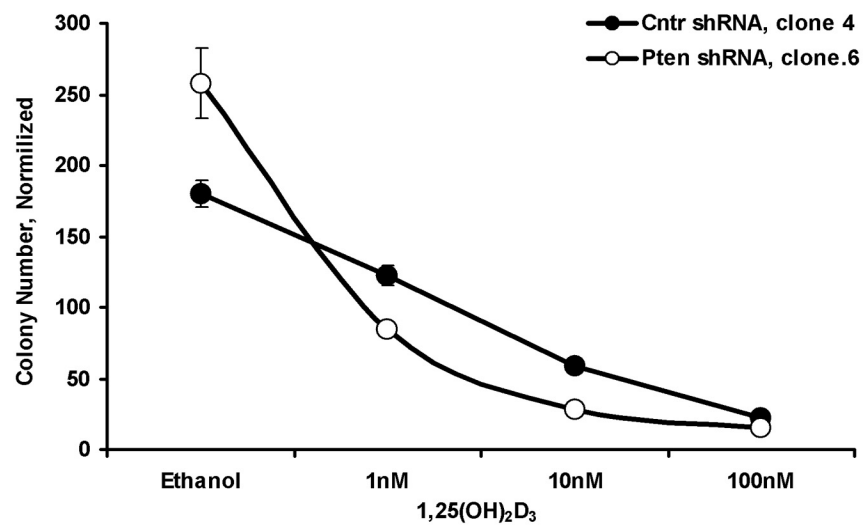


C

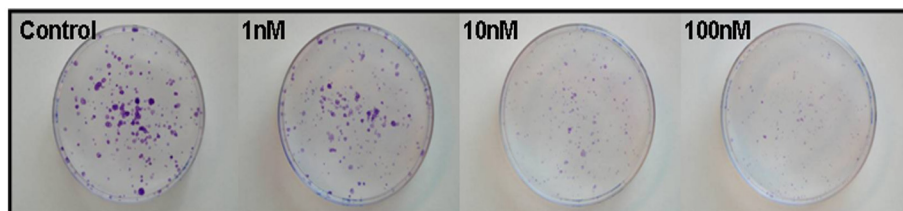


Supplemental Figure 6

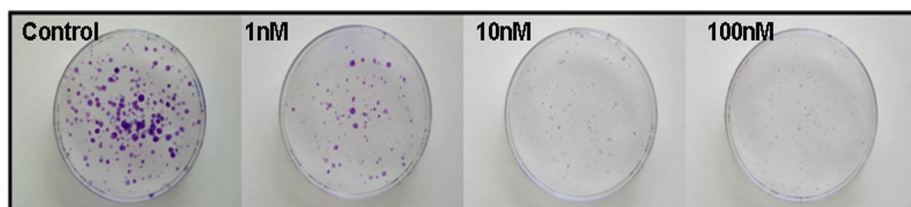
A



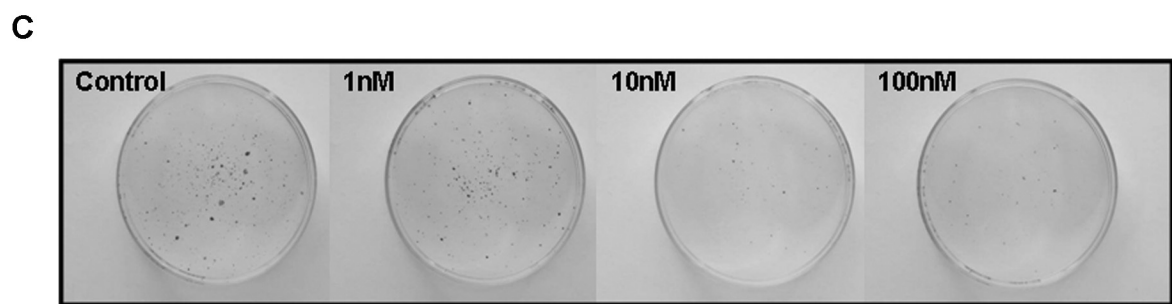
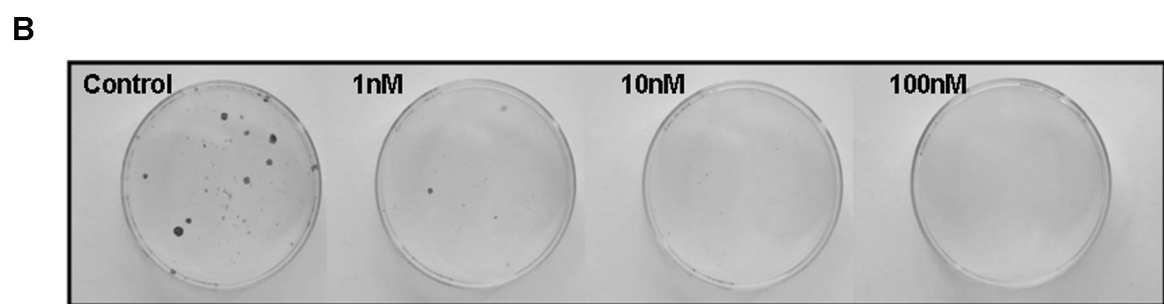
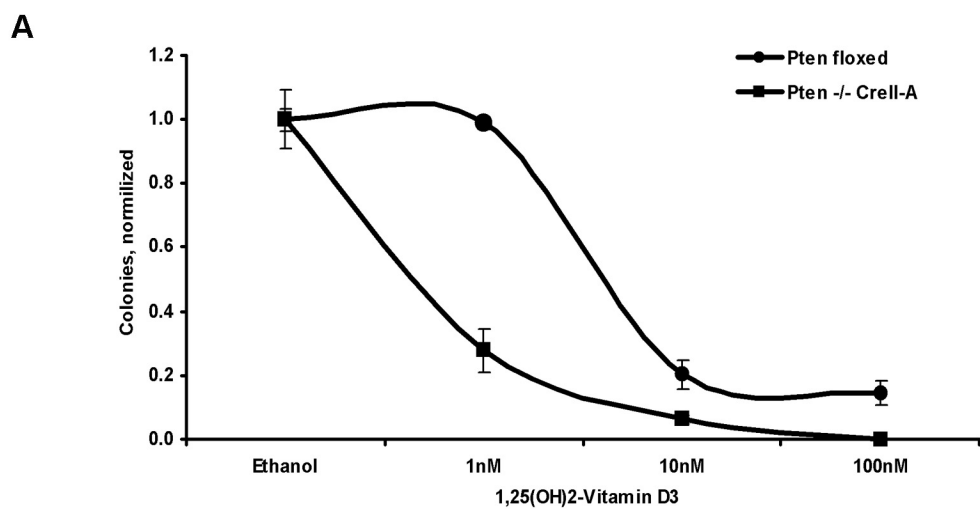
B



C



Supplemental Figure 7



Supplemental Information.

Materials and Methods:

Real-time PCR for evaluation of Pten deletion. Genomic DNA was extracted from MPEC and real-time PCR was performed for Pten lacking exon 5, Pten WT and Pten Lox site using iCycler (Bio-Rad) with QuantiTect SYBR Green PCR kit (QIAGEN) as previously described(24). PCR reaction consisted of 50 cycles of 15 seconds at 94°C, 30 seconds at 57°C, and 45 seconds at 72°C. Reaction mixtures without genomic DNA were used as negative controls.

Immunoblot:. Plates were washed twice with cold PBS, lysis buffer (5mM EDTA, 150mM NaCl, 50mM Tris-HCl, 1% NP-40) containing freshly added protease inhibitor cocktail (PIC), 1mM DTT, 1mM Na₃VO₄ and 5mM NaF was added to the plates. Plates were placed on ice for 20min with tilting every 5min. Cells were scrapped and collected into eppendorf tubes, centrifuged at 13000rpm for 15min at 4°C. Supernatants were collected and stored at -80°C. 50-100µg of protein was subjected to electrophoresis on SDS-PAGE gels. Proteins were transferred onto prewetted Hybond-P PVDF (polyvinylidene difluoride) membrane according to manufactures' protocol (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were incubated with appropriate dilutions of Pten (sc-7974) (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were used. ECL Plus Western Blotting Detection System kit (Amersham-Pharmacia) was used for detection of proteins.

Clonogenic survival assays. Cells were plated in 6-cm diameter dishes at 2000 or 4000 cells per plate in medium containing 1nM, 10nM or 100nM 1,25(OH)₂D₃ or vehicle (ethanol, 1x = 0.1% V/V) and allowed to grow for 7 days. Platings were performed in quadruplicate. After incubation, cells were fixed with 10% methanol–10% acetic acid and stained with a 0.4% solution of crystal violet. Colonies with >50 cells were counted. Error bars represent SD.

Supplemental Figure Legends:

Supplemental Figure 1. LY294002 synergizes with 1,25(OH)₂D₃ to inhibit growth of LNCaP, DU145 cells and Pten^{-/-} MPEC.

Growth inhibition of LNCaP cells **(A)**, DU145 cells **(B)** and Pten^{-/-} MPEC (clone 11) **(C)** treated with 1,25(OH)₂D₃ in presence or absence of LY294002. Cells were grown and treated as described in Materials and Methods. Viable cells were counted with a hemacytometer after trypan blue exclusion and presented as means for the triplicates +SD. Means without a common letter are significantly different by ANOVA ($P < 0.05$).

Supplemental Figure 2. Inhibition of AKT with API-2 or GSK690693 synergizes with 1,25(OH)₂D₃ to inhibit growth of prostate cancer cells.

(A) Growth inhibition of DU145 cells treated with 1,25(OH)₂D₃ in presence or absence of API-2. **(B)** Growth inhibition of WFU275Ca cells treated with 1,25(OH)₂D₃ in presence or absence of API-2. **(C)** Growth inhibition of DU145 cells treated with 1,25(OH)₂D₃ in presence or absence of GSK690693. **(D)** Growth inhibition of WFU273Ca cells treated with 1,25(OH)₂D₃ in presence or absence of GSK690693. Cells were grown and treated as described in Materials and Methods. Viable cells were counted with a hemacytometer after trypan blue exclusion and presented as means for the triplicates +SD. Means without a common letter are significantly different by ANOVA ($P < 0.05$).

Supplemental Figure 3. API-2 synergizes with 1,25(OH)₂D₃ to inhibit growth of human primary prostate cancer cell strain WFU273Ca.

(A) Growth inhibition of WFU273Ca cells in response to API-2 and 1,25(OH)₂D₃ alone or in combination. Cells were grown treated as described in Materials and Methods. Viable cells were counted with a hemacytometer after trypan blue exclusion and presented as means for the triplicates +SD. Means without a common letter are significantly different by ANOVA ($P < 0.05$). **(B)** Number of viable cells normalized to control presented as means \pm SD.

Supplemental Figure 4. 1,25(OH)₂D₃ and API-2 induce senescence in cooperative manner in DU145 cells. Cells were grown, treated and (SA)- β -galactosidase activity was evaluated as described in Materials and Methods. Representative photographs taken at 20X magnification are demonstrated.

Supplemental Figure 5.

(A) Immunoblot analysis of Pten expression in WFU3 MPEC infected with Pten shRNA or scrambled shRNA as described in Materials and Methods.

(B) PCR analysis of Pten deletion by self-deleting Cre-recombinase lentivirus in Pten^{lox/lox} MPEC. Pten^{lox/lox} MPEC isolated from 8 weeks old Pten lox/lox Cre-recombinase negative animals and infected with self-deleting Cre-recombinase lentivirus to generate Pten^{-/-} MPEC.

(C) Immunoblot analysis of Pten deletion by self-deleting Cre-recombinase lentivirus in Pten^{lox/lox} MPEC. Pten^{lox/lox} MPEC isolated from 8 weeks old Pten^{lox/lox} Cre-recombinase negative animals and infected with self-deleting Cre-recombinase lentivirus.

Supplemental Figure 6. *In vitro* suppression of Pten with shRNA doesn't affect clonogenic survival of cells in response to 1,25(OH)₂D₃.

Clonogenic survival **(A)** and representative photographs of clonogenic survival of WFU3 MPEC (Pten shRNA, clone 6) **(B)** and WFU3 MPEC scrambled shRNA (clone 4) **(B)** treated with increasing concentrations of 1,25(OH)₂D₃. Cells were plated at 2000 cells per 60mm tissue culture dish. Values are average for quadruplicates±SD are demonstrated.

Supplemental figure 7. *In vitro* loss of Pten doesn't affect clonogenic survival of cells in response to 1,25(OH)₂D₃. Clonogenic survival **(A)** and representative photographs of clonogenic survival of Pten^{lox/lox} MPEC **(B)** and respective Pten^{-/-} MPEC (Pten^{lox/lox} MPEC infected with self-deleting Cre-recombinase) **(C)** treated with increasing concentration of 1,25(OH)₂D₃. Pten^{lox/lox} MPEC were plated at 2000 cells per 60mm tissue culture dish and at 4000 cells per dish for Pten^{-/-} MPEC. Values are average for quadruplicates±SD.